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Date: 2/5/08

Zhibin Ren, Reg. No. 47,897

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Before the Board of Patent Appeals and Interferences

Applicant: Mark E. Cook et al. Group Art Unit: 1616

Appl. No.: 10/756,719 Examiner: Ernst V. Arnold

Filed: January 13, 2004 Docket No.: 960296.00108

For: METHOD OF TREATING TYPE III HYPERSENSITIVE REACTION RELATED DISEASES AND CONDITIONS

TRANSMITTAL OF BRIEF ON APPEAL

Mail Stop Appeal Brief - Patent
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant hereby submits an Appeal Brief in support the Notice of Appeal filed on December 13, 2007.

The $510.00 fee for filing an Appeal Brief and any other fees due should be charged to Deposit Account No. 17-0055.

Respectfully submitted,

By:

Zhibin Ren
Reg. No. 47,897
Attorney of Record
Quarles & Brady LLP
411 E. Wisconsin Avenue
Milwaukee, WI 53202-4497
(414) 277-5633

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PATENT

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APPELLANT'S BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellant, Mark E. Cook et al., having herewith filed a timely Notice of Appeal in the above-identified patent application, also hereby submits this Brief on Appeal.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Wisconsin Alumni Research Foundation, a non-stock, non-profit Wisconsin corporation located at 614 Walnut Street, Madison, WI, 53726, as

evidenced by the assignment recorded at Reel/Frame No. 014647/0115.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF CLAIMS

Claims 3 and 5-16 are pending and claims 1, 2, and 4 are canceled in the subject patent application. Claims 3 and 5-16 stand rejected (they have been rejected at least twice). This appeal is taken with respect to claims 3 and 5-16, which are set forth in Appendix A hereto.

IV. STATUS OF AMENDMENTS

All amendments have been entered. Although the advisory action dated November 27, 2007 indicates that "the proposed amendment(s) will not be entered" in connection with the response filed on October 31, 2007, appellant notes that said response did not introduce any amendment to the pending claims.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

A. RELATION OF INDEPENDENT CLAIM 3 TO THE DESCRIPTION.

3. A method for treating rheumatoid arthritis in a human or non-human animal in need thereof, the method comprising the steps of (paragraph [0008], lines 1-2; and paragraph [00010], lines 1-3 and 17-19):

administering to the human or non-human animal a composition that consists of a conjugated linoleic acid (CLA) and one or more carriers (paragraph [0008], lines 2-5; paragraph [00010], lines 2-3; and paragraphs [00017]-[00019]) wherein the

CLA is in an amount effective to reduce joint inflammation in the human or non-human animal (paragraph [00023], lines 9-13; paragraph [00025]; paragraphs [0008] and [00009]; and Fig. 1); and

observing an improvement in joint redness and swelling in the human or non-human animal (paragraph [00023], lines 9-13; paragraph [00025]; paragraphs [0008] and [00009]; and Fig. 1).

VI. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

A. THE PRIOR ART REJECTION UNDER 35 U.S.C. 103(a).

Claims 3 and 5-16 stand rejected under 35 U.S.C. 103(a) as being obvious over Cook et al. (US 6,395,782; hereinafter "Cook et al.") in view of Horrobin et al. (US 6,245,811; hereinafter "Horrobin et al.") (office action dated August 14, 2007, pages 2-6).

VII. ARGUMENT

A. CLAIMS 3 AND 5-16 ARE NOT OBVIOUS UNDER 35 U.S.C. 103(A) OVER COOK ET AL. IN VIEW OF HORROBIN ET AL.

1. The Examiner's rejection.

The Examiner's rejection includes two parts: (i) the use of conjugated linoleic acid to reduce joint redness and swelling in a rheumatoid arthritis patient is obvious over Cook et al. and (ii) the use of an ester of conjugated linoleic acid to reduce joint redness and swelling in a rheumatoid arthritis patient is obvious over Cook et al. in view of Horrobin et al.

With respect to (i), the Examiner alleges that Cook et al. teach methods of extending the survival time of a human or non-human animal having a disease, thus in need of treatment, characterized by autoimmune complexes by administering an effective amount of conjugated linoleic acid and that the methods are suitable for treating rheumatoid arthritis (office action dated August 14, 2007, page 3, lines 1-5). The Examiner further alleges that Cook et al. teach various other elements of the pending claims (office action dated August 14, 2007, page 3, lines 5-11). However, Cook et al. do not teach the element of "improvement in joint redness and swelling" recited in independent claim 3. In this regard, the Examiner alleges administering conjugated linoleic acid to the patient would reduce the symptoms of rheumatoid arthritis including joint inflammation resulting in improvement of joint redness and swelling (office action dated August 14, 2007, page 5, lines 14-20).

With respect to (ii), the Examiner states that Cook et al. do not expressly teach a method wherein the conjugated linoleic acid is an ester of a conjugated linoleic acid (office action dated August 14, 2007, page 4, lines 7-8). However, the Examiner alleges that Horrobin et al. suggest that esters of conjugated linoleic acid can be used in a method of treating rheumatoid arthritis, citing column 14, lines 55-62; column 15, line 1; and claims 1, 10, and 28 (office action dated August 14, 2007, page 4, line 12 to page 4, line 4). In the Examiner's opinion, it would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to use an ester of conjugated linoleic acid suggested by Horrobin et al. in the method of treating rheumatoid arthritis taught by Cook et al. (office action dated August 14, 2007, page 4 lines 11-17).

According to the Examiner, it is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose (office action dated August 14, 2007, page 4, lines 17-21, citing *In re Kerkhoven*, 626 F.2d 846, 850 (CCPA 1980)).

2. Summary of appellant's arguments.

With respect to (i) noted above, the use of conjugated linoleic acid to reduce joint redness and swelling in a rheumatoid arthritis patient is not obvious over Cook et al. Cook et al. did not teach that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis patients. What Cook et al. taught was that administering conjugated linoleic acid to a human or non-human animal having a condition associated with the existence of autoimmune complexes (rheumatoid arthritis is an example) can extend the survival time or reduce body weight wasting of the human or non-human animal. In this regard, the Examiner stated that administering conjugated linoleic acid to a rheumatoid arthritis patient would reduce joint redness and swelling. If the Examiner meant that conjugated linoleic acid would inherently reduce joint redness and swelling, this is a legal mistake in that what is inherent in the prior art, if not known at the time of the invention, cannot form a proper basis for rejecting the claimed invention as obvious under § 103. *See In re Shetty*, 566 F.2d 81, 86 (CCPA 1977). If the Examiner meant that the teaching of Cook et al. on extending the survival time and reducing body wasting would make it obvious that conjugated linoleic acid would be able to reduce joint redness and swelling, the evidence on the record suggests

the opposite. See Yang et al., Immunopharmacology and Immunotoxicology 2000, 22:433-449 (hereinafter "Yang et al."); Sugano et al., Lipids, 1998, 33:521-527 (hereinafter "Sugano et al."); and Yamasaki et al., J. Nutr., 2003, 133:784-788 (hereinafter "Yamasaki et al.").

With respect to (ii) noted above, the use of an ester of conjugated linoleic acid to reduce joint redness and swelling in a rheumatoid arthritis patient is not obvious over Cook et al. in view of Horrobin et al. Horrobin et al. did not teach or suggest that esters of conjugated linoleic acid can be used to treat rheumatoid arthritis, much less to reduce joint redness and swelling in particular. Therefore, what the combination of Cook et al. and Horrobin et al. would suggest is to use an ester of conjugated linoleic acid to extend the survival time and reduce body weight wasting. As provided above, whether an ester of conjugated linoleic acid would inherently reduce joint redness and swelling is irrelevant in an obvious inquiry as this effect was not recognized in the prior art at the time the application was filed. Also as provided above, the evidence on the record supports that an ester of conjugated linoleic acid is unlikely to reduce joint redness and swelling even assuming it can extend the survival time and reduce body weight wasting.

3. The use of conjugated linoleic acid to reduce joint redness and swelling in a rheumatoid arthritis patient is not obvious over Cook et al.

(a) Cook et al. do not teach that conjugated linoleic acid can reduce joint redness and swelling.

Cook et al. teach that administering conjugated linoleic acid to a human or non-human animal having a condition associated

with the existence of autoimmune complexes can extend the survival time and reduce body weight wasting of the human or non-human animal (see e.g., column 2, line 63 to column 3, line 30). Cook et al. teach that such conditions include rheumatoid arthritis (column 3, lines 37-43 and 50). However, Cook et al. do not teach specifically that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis, which is an element of pending independent claim 3.

(b) It is irrelevant whether conjugated linoleic acid inherently reduces joint redness and swelling.

In response to the applicant's argument that Cook et al. did not teach that conjugated linoleic acid can reduce joint redness and swelling, the Examiner asserted that treatment of a condition does reduce the symptoms of the condition and thus administering conjugated linoleic acid would reduce joint redness and swelling (office action dated August 14, 2007, page 5, lines 17-20). It is not clear what the Examiner meant by this assertion because it is not uncommon that a treatment of a condition reduces some but not all symptoms of the condition. For example, a patient suffering from a common cold may experience fever, pain, nasal congestion, excess cough and other symptoms. Acetaminophen can reduce fever and pain but not other symptoms. Similarly, decongestants will relive congestion but not other symptoms. Therefore, just because Cook et al. taught that conjugated linoleic acid can extend the survival time and reduce body weight wasting does not mean that it can also reduce joint redness and swelling. In fact, in the case of the immune complex-related disease Cook et al. used as an example to demonstrate the effectiveness of conjugated linoleic acid for extending survival time and reducing body weight wasting (systemic lupus

erythematosus), the evidence on the record shows that conjugated linoleic acid promotes rather than reduces some other symptoms of the disease (see discussion of Yang et al. in subsection VII.A.3(c) below).

If by the assertion noted above the Examiner meant administering conjugated linoleic acid would inherently reduce joint redness and swelling, this is legally incorrect in that what is inherent in the prior art, if not known at the time of the invention, cannot form a proper basis for rejecting the claimed invention as obvious under § 103. *See In re Shetty*, 566 F.2d 81, 86 (CCPA 1977). Since Cook et al. do not teach and it was not known in the art at the time the application was filed that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis, Cook et al. cannot form the basis for the obviousness rejection in this regard.

(c) It is not obvious to one of ordinary skill in the art that conjugated linoleic acid can reduce joint redness and swelling based on the teaching of Cook et al.

If by the assertion noted above (office action dated August 14, 2007, page 5, lines 17-20) the Examiner meant that the teaching of Cook et al. makes it obvious to one of ordinary skill in the art that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis, appellant notes that the evidence on the record does not support this conclusion.

First of all, the only experimental evidence on the record (Yang et al.) shows that even though conjugated linoleic acid can extend the survival time and reduce body weight wasting in a subject having a disease associated with the existence of autoimmune complexes as taught by Cook et al., the same agent promotes rather than reduces certain symptoms of the disease.

Cook et al. teach that conjugated linoleic acid extends the survival time and reduces body weight wasting in mice having systemic lupus erythematosus (column 4, line 40 to column 5, line 34). However, Yang et al. show with the same mice that conjugated linoleic acid promoted the early onset of proteinuria, a symptom of systemic lupus erythematosus (Fig. 3 and related text of Yang et al.).

Furthermore, at the time the application was filed, there was evidence in the art that conjugated linoleic acid increases antibody production in the body. Given that rheumatoid arthritis depends on the formation and deposit of antibody/antigen immune complexes, it is counterintuitive that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis in that the increased antibody production may promote the formation and deposit of antibody/antigen immune complexes and thus aggravate the symptom. As discussed in the present application (e.g., paragraphs [00010] and [00011]), type III hypersensitivity such as rheumatoid arthritis is caused by antibody/antigen immune complex deposition, leading to tissue damage and inflammatory reactions. In the case of rheumatoid arthritis, the immune complex is between type II collagen and the auto-antibodies to type II collagen (see e.g., the example section of the application in which anti-type II collagen antibody was injected to induce rheumatoid arthritis). Sugano et al. have shown that conjugated linoleic acid increases immunoglobulin (antibody) production. Yamasaki et al. have also shown that conjugated linoleic acid increases immunoglobulin (antibody) production. Importantly, Yang et al. showed that conjugated linoleic acid promoted the earlier appearance of antinuclear antibodies and the symptom of proteinuria caused by the deposit of the antinuclear antibody immune complexes in the kidney (Figs. 3 and 4 of Yang et

al.). Therefore, one of ordinary skill in the art is more likely to conclude that conjugated linoleic acid extends the life span and reduces body weight wasting in rheumatoid arthritis by other mechanisms such as improving feed behavior (US patent 5,428,072) rather than reducing the formation and deposit of antibody/antigen immune complexes, which underlies joint redness and swelling. Accordingly, one of ordinary skill in the art would not conclude with a reasonable likelihood of success based on the teachings of Cook et al. that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis.

For all of the above reasons, the teaching of Cook et al. on conjugated linoleic acid's ability to extend the survival time and reduce body weight wasting in rheumatoid arthritis does not suggest that the agent can reduce joint redness and swelling.

4. The use of an ester of conjugated linoleic acid to reduce joint redness and swelling in a rheumatoid arthritis patient is not obvious over Cook et al. in view of Horrobin et al.

(a) Horrobin et al. do not teach that an ester of conjugated linoleic acid can be used to treat rheumatoid arthritis, much less to reduce joint redness and swelling in particular.

Contrary to the Examiner's position, Horrobin et al. do not teach that an ester of conjugated linoleic acid can be used to treat rheumatoid arthritis. What Horrobin et al. teach are that an ester of GLA, DGLA, SA and EPA can be used to treat rheumatoid arthritis and an ester of conjugated linoleic acid can be used to treat cancer, cardiovascular disease, metabolism diseases, to promote growth of protein-containing tissues and treat related diseases, and to act as an antioxidant and treat related diseases.

Horrobin et al. is not about new activities of any compound. Rather, it is about combining two or more known bioactive compounds into the same molecule to achieve increased lipophilicity (for passing lipid barriers in the body) as well as additive or maybe even synergistic effect (column 1, lines 13-64). In this regard, it relies on compounds with known bioactivities. At the time the Horrobin et al. application was filed (August 18, 1999), conjugated linoleic acid was known to be effective for treating some other diseases, but not rheumatoid arthritis. The specification of Horrobin et al. is consistent with this. For example, Horrobin et al. mentions the treatment of rheumatoid arthritis in connection with one or more of GLA, DGLA, SA and EPA, but not conjugated linoleic acid (see column 13, lines 12-16 and 30-31). The only activities specifically mentioned in connection with conjugated linoleic acid are, as known in the art at that time, treating or preventing cancer, treating or preventing cardiovascular disease, treating metabolism diseases, promoting growth of protein-containing tissues, and acting as an antioxidant (see column 6, lines 35-39 and column 14, lines 6-10). Horrobin et al. did not present any data to indicate that they discovered a new activity of conjugated linoleic acid for treating rheumatoid arthritis.

The sections of Horrobin et al. cited by the Examiner (column 14 at lines 55-62, column 15 at line 1, and claims 1, 10, and 28) list a plurality of compounds including, among others, GLA, DGLA, SA, EPA, DHA, and conjugated linoleic acid (CLA) for treating a plurality of diseases including, among others, impotence, male pattern baldness, renal and urinary tract disorders, cancer, cardiovascular disease/disorder, and rheumatoid arthritis (column 14 at lines 55-62 and 66, column 15 at lines 1 and 9, and claims 1, 10, and 28). The Examiner seems

to read the above sections to mean that any of the compounds listed can treat any of the diseases listed. However, this is not the case. For example, EPA and DHA which are among the bioactive compounds listed are not effective for treating impotence and male pattern baldness which are among the diseases listed, although they are effective for treating some other listed diseases such as renal and urinary tract disorders. Similarly, conjugated linoleic acid was not known to be effective for treating rheumatoid arthritis but known to be effective for treating certain other listed diseases such as cancer and cardiovascular disease. Therefore, the reasonable way of reading the sections cited by the Examiner would be that a compound can be used to treat one or more diseases listed there for which the compound was known to be effective at the time the Horrobin et al. application was filed but not that a compound can be used to treat all of the diseases listed. This reasonable way of reading the sections does not violate any claim construction rules with respect to claims 1, 10, and 28 cited by the Examiner. Accordingly, given that Horrobin et al. did not specifically disclose or present any data to indicate that they had identified a new activity of conjugated linoleic acid for treating rheumatoid arthritis, one of ordinary skill in the art would understand that conjugated linoleic acid (CLA) was listed among the bioactive compounds for its known effects on cancer and cardiovascular disease, but not rheumatoid arthritis.

For the same reasons, one of ordinary skill in the art would not infer from Horrobin et al., much less with a reasonable likelihood of success as required by law, that an ester of conjugated linoleic acid can be used to treat rheumatoid arthritis, much less to reduce joint redness and swelling in particular. If this were to be inferred, it would also mean that

an ester of EPA or DHA would be able to treat impotence and male pattern baldness, which is not consistent with the understanding of the art (see details discussed above). Therefore, Horrobin et al. do not teach and one of ordinary skill in the art would not reasonably infer from Horrobin et al. that an ester of conjugated linoleic acid can be used treat rheumatoid arthritis, much less to reduce joint redness and swelling in particular.

(b) The combination of Cook et al. and Horrobin et al. do not suggest that an ester of conjugated linoleic acid can be used to reduce joint redness and swelling.

The combination of Cook et al. and Horrobin et al. do not suggest that an ester of conjugated linoleic acid can be used to reduce joint redness and swelling in rheumatoid arthritis. The Examiner appears to agree with applicant that conjugated linoleic acid esters were not known to be effective against rheumatoid arthritis at the time the Horrobin application was filed (office action dated August 14, 2007, page 6, lines 1-6). However, the Examiner asserts that Cook et al. specifically teach treatment of rheumatoid arthritis with conjugated linoleic acid, and because Horrobin et al. teach the use of esters of conjugated linoleic acid, the use of the esters for arthritis treatment would be obvious (office action dated August 14, 2007, page 6, lines 5-8). As discussed above, Cook et al. do not teach and it is not obvious from Cook et al. that conjugated linoleic acid can reduce joint redness and swelling. Therefore, what the combination of Cook et al. and Horrobin et al. would suggest would be to use an ester of conjugated linoleic acid to extend the survival time and reduce body weight wasting. Similar to the discussion above in connection with Cook et al., whether an ester of conjugated linoleic acid would inherently reduce joint redness and swelling

is irrelevant in an obvious inquiry as this effect was not recognized in the prior art at the time the application was filed. Furthermore, the evidence on the record supports that conjugated linoleic acid and thus an ester of conjugated linoleic acid is unlikely to reduce joint redness and swelling even assuming it can extend the survival time and reduce body weight wasting.

With respect to the Examiner's assertion that it is *prima facie* obvious to combine two compositions (conjugated linoleic acid in Cook et al. and an ester of conjugated linoleic acid in Horrobin et al.) each of which is taught by the prior art to be useful for the same purpose (office action dated August 14, 2007, page 4, lines 17-21), appellant notes that it is not the case that Cook et al. and Horrobin et al. teach two compositions each of which is useful for the same purpose, especially for the particular purpose of reducing joint redness and swelling in rheumatoid arthritis. It is acknowledged that a reference is good not only for what it teaches directly but also for what one of ordinary skill in the art might reasonably infer from the teachings (office action dated August 14, 2007, page 4, line 21 to page 5, line 5). However, as discussed in detail above, Cook et al. do not teach and one of ordinary skill in the art would not reasonable infer that conjugated linoleic acid can reduce joint redness and swelling in rheumatoid arthritis. Similarly, Horrobin et al. do not teach and one of ordinary skill in the art would not reasonably infer from Horrobin et al. that an ester of conjugated linoleic acid can be used to either treat rheumatoid arthritis or reduce joint redness and swelling in rheumatoid arthritis. Therefore, the pending claims are not *prima facie* obvious as alleged by the Examiner in the office action dated August 14, 2007 at page 4, lines 17-21.

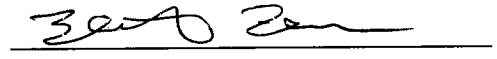
5. Summary.

Neither Cook et al. nor Horrobin et al. teach or suggest, either alone or in combination, that conjugated linoleic acid or an ester thereof can relieve the specific symptom of joint redness and swelling of rheumatoid arthritis. The evidence on the record shows that conjugated linoleic acid aggravates other symptoms (Yang et al.) or may potentially aggravate joint redness and swelling (Sugano et al. and Yamasaki et al.). When Yang et al., Sugano et al., and Yamasaki et al. are considered together with Cook et al. and Horrobin et al., it would not have been obvious to one of ordinary skill in the art that conjugated linoleic acid can be used to reduce joint redness and swelling in rheumatoid arthritis. At the very least, there would have been sufficient doubt in the mind of one of ordinary skill in the art so that he or she would not have concluded that there would be a reasonable likelihood of success for using conjugated linoleic acid to reduce joint redness and swelling in rheumatoid arthritis.

VIII. CONCLUSION

In view of the above, appellant respectfully requests the Board to reverse the Examiner on the obviousness rejection regarding claims 3 and 5-16.

Respectfully submitted,

By: 

Zhibin Ren
Reg. No. 47,897
Quarles & Brady LLP
411 East Wisconsin Avenue
Milwaukee, WI 53202-4497
(414) 277-5633
Attorney of Record

APPENDIX A

Claims on Appeal in Patent Application No. 10/756,719

3. A method for treating rheumatoid arthritis in a human or non-human animal in need thereof, the method comprising the steps of:

administering to the human or non-human animal a composition that consists of a conjugated linoleic acid (CLA) and one or more carriers wherein the CLA is in an amount effective to reduce joint inflammation in the human or non-human animal; and

observing an improvement in joint redness and swelling in the human or non-human animal.

5. The method of claim 3, wherein the CLA is selected from a free conjugated linoleic acid, an ester of a conjugated linoleic acid, a non-toxic salt of a conjugated linoleic acid, an active isomer of a conjugated linoleic acid, an active metabolite of a conjugated linoleic acid, and a mixture thereof.

6. The method of claim 5, wherein the free conjugated linoleic acid is selected from an 18:2(9c,11t) isomer, an 18:2(9t,11c) isomer, an 18:2(10c,12t) isomer and an 18:2 (10t,12c) isomer.

7. The method of claim 3, wherein the animal is selected from a mammal and an avian.

8. The method of claim 7, wherein the mammal is selected from a human, a non-human primate, a horse, a canine, a feline, a rodent, a porcine, a bovine, a caprine and an ovine.

9. The method of claim 8, wherein the mammal is selected from a human, a horse, a canine and a feline.

10. The method of claim 9, wherein the mammal is a human.

11. The method of claim 3, wherein the administering step comprises a method selected from oral delivery, intramuscular injection, intravenous injection, transdermal delivery, transmucosal delivery and parenteral delivery.

12. The method of claim 11, wherein the administering step comprises oral delivery.

13. The method of claim 12, wherein the CLA is added to a food and the food is consumed by the animal.

14. The method of claim 13, wherein the food contains 0.01% to 5% of CLA by weight of the food.

15. The method of claim 14, wherein the food contains 0.05% to 2% of CLA by weight of the food.

16. The method of claim 3, wherein the CLA is administered in a dosage of between about 0.001 g/kg and 1 g/kg body weight of the animal.

APPENDIX B

Factual Evidence in Application No. 10/756,719

Submitted as part of Appeal Brief.

(1) Yang et al., Immunopharmacology and Immunotoxicology 2000, 22:433-449 (copy included in the response filed on April 17, 2006).

(2) Sugano et al., Lipids, 1998, 33:521-527 (copy included in the response filed on September 15, 2006).

(3) Yamasaki et al., J. Nutr., 2003, 133:784-788 (copy included in the response filed on September 15, 2006).

APPENDIX C

Related Proceedings in Application No. 10/756,719

Submitted as part of Appeal Brief.

(None)

QBACTIVE\6058225.2

IMMUNOPHARMACOLOGY AND IMMUNOTOXICOLOGY, 22(3), 433–449 (2000)

DIETARY CONJUGATED LINOLEIC ACID PROTECTS AGAINST END STAGE DISEASE OF SYSTEMIC LUPUS ERYTHEMATOSUS IN THE NZB/W F1 MOUSE

Mingder Yang
Department of Animal Science, University of Wisconsin-Madison

Michael W. Pariza
Department of Food Microbiology and Toxicology, University of Wisconsin-Madison

Mark E. Cook*
Department of Animal Science and Department of Food Microbiology and Toxicology, University of Wisconsin-Madison

Abstract

Conjugated linoleic acid (CLA) is a naturally occurring fatty acid with anti-carcinogenic, anti-atherosclerotic and immune-enhancing activities. Dietary CLA accelerated the onset of proteinuria in autoimmune-prone NZB/W F1 mice but did not affect anti-DNA antibody production. Body weight of the CLA group was decreased compared to the control group at the time proteinuria first developed. CLA group also had slightly earlier mortality than control fed mice, however the mean days of survival did not differ between CLA and control fed mice. Body weight loss between proteinuria onset and death was approximately twice as much in the control group as in the CLA group. Moreover, duration between proteinuria and death was longer in the CLA than in the control group. Our data suggested that dietary CLA may accelerate the autoimmune symptoms of NZB/W F1 mice, however, CLA protected against the disease related body weight loss and prolonged survival after proteinuria.

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometrical isomers of linoleic acid (1). These naturally occurring fatty acids are found in beef and dairy products due to ruminal isomerization of linoleic acid (2). Recent discoveries have shown that CLA has anti-carcinogenic activity (1), anti-atherosclerotic activity (3) and alters body composition by reducing body fat and increasing lean body mass (4). CLA was also shown to modulate the immune response by increasing lymphocyte cytotoxic activity, macrophage killing ability (5, 6) and lymphocyte blastogenesis (6, 7). Since CLA modulates immune reactivity, will it predispose animals prone to immune disorders to disease?

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease caused by defects in immune regulation that result in hyperactive T and B lymphocytes. Unlike organ-specific autoimmune diseases, a systemic autoimmune disease causes widespread tissue damage by cell-mediated immune responses, autoantibodies or immune complexes. A hallmark of SLE pathogenesis is the presence of serum autoantibodies against nuclear components as a result of immune dysregulation. For example, IgG autoantibodies to DNA are responsible for the formation of immune complexes in SLE glomerulonephritis (8, 9). The immune complexes are deposited along the wall in the small blood vessel of kidney, resulting in glomerulonephritis. Glomerular leakage of plasma proteins makes proteinuria an indicator of kidney damage originated from autoantibodies. Researchers have shown in SLE patients and rodent models that n-3 fatty acids and decreased calorie intake were beneficial in alleviating the clinical signs of disease (10-13).

Due to CLA's ability to modulate immune system, the evidence of fatty acid involvement in autoimmune disorders and the increased availability of highly enriched CLA supplements, it seemed imperative to determine the influence of CLA on autoimmune disorders. The objective of this research was to determine

the influence of dietary CLA on immune related disorders in the SLE-prone NZB/W F1 mice.

RESEARCH DESIGN AND METHODS

Materials. Conjugated linoleic acid (Natural Lipids Inc, Hovdebygda, Norway) contained 90% CLA (CLA-90) with the following C18:2 isomer distribution: 43.5% t10,c12, 41.9% c9,t11 and t9,c11, 1.5% t9,t11 and t10,t12, 0.9% c9,c11, 0.9% c10,c12. Other fatty acids in CLA-90 were 5.6% oleate, 1.4% palmitate, 0.5% linoleate, 0.4% stearate, and 3.4% unidentified compounds. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri) unless specified.

Diet. Semi-purified powdered diet (TD94060, 99% basal mix, Harlan-Teklad, Madison, WI) was mixed with 0.5% oil (either CLA or corn oil) and 0.5% sucrose by weight, such that the final diet contained either 5.5% corn oil (control group) or 5% corn oil plus 0.5% CLA (CLA group) (4). Diets were prepared fresh every other week and stored at 4 ^{o}C. Diets and water were provided *ad libtum*.

Animals. NZB/W F1, the offspring of New Zealand Black mice and New Zealand White mice, is a well-established animal model for human SLE study (9). Twenty female NZB/W F1 mice were obtained at four weeks of age from Harlan-Sprague Dawley (Madison, WI) and housed in a temperature and humidity controlled room with 12 hour light/dark cycle. Two mice were housed in a cage and given a pelleted chow for a week before they were randomly assigned to treatment diet (10 per diet). Protocols for animal care and use were approved by the Research Animal Resources Center of University of Wisconsin-Madison.

Feed Intake and Body Weight. Diet intake was recorded every other day in the first three weeks of the trial to determine intake and efficiency of conversion of

diet into body mass (feed efficiency). Weekly body weight and survival of the mice were also recorded from the beginning of the experiment to the time of death. For humane reasons, mice were euthanized when they became lethargic and stopped eating.

Urine Collection and Proteinuria Assay. Mouse urine was collected by using metabolic cages every other week before 28 weeks of age and weekly thereafter. Each mouse was housed in a single metabolic cage for 3 hours to collect urine. A 20 μl urine sample was diluted four times with distilled water. In a 96 well plate, 20 μl of diluted urine sample was placed in a well, and 200 μl of diluted Bio-Rad protein assay reagent was then added to each well. Plates were mildly shaken for 5 min and the color reactions were read at 600 nm with a microtiter plate reader (Autoreader EL310, Bio-tek Instrument). Duplicate samples were applied in microtiter plates and bovine γ-globulin was used as positive control and also to create a standard curve.

Serum Collection and ELISA for Anti-DNA Antibodies. Blood was drawn retro-orbitally every other week after mice were placed on their dietary treatments. Serum was separated by centrifugation and stored at -70 ^{0}C until assayed.

Enzyme-linked immunosorbent assay (ELISA) for serum anti-single strand (ss) or double strand (ds) DNA Antibody (Ab) was determined as follows: Immulon II HB plates (Dynex, Chantilly, VA), were coated with 50 μl ds calf thymus DNA (50 μg/ml) in coating buffer (50 mM sodium bicarbonate, pH 8.5). Plates were gently shaken for one hour and placed on the bench top overnight. Plates were then washed with phosphate buffered saline (PBS)-tween 20 (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4 and 0.5 ml Tween 20 in one liter of deionized distilled water) solution four times. One hundred and fifty μl of 1% bovine serum albumin (BSA) in PBS was then added to the wells and gently

shaken for one hour. Plates were emptied by inversion. Serum samples were diluted 1: 80 with 1% BSA solution, and 50 µl of diluted serum was added to each well and plates were shaken for one hour. Plates were then washed 4 times and drained as described above. Horseradish peroxidase conjugated goat anti-mouse IgG (γ-chain specific detection antibody) was diluted 1: 2,000 with 1% BSA in PBS. To each well, 50 µl of diluted detection antibody was added. After one hour incubation and shaking, an extensive wash was applied to remove unbound detection antibody. One hundred and twenty-five µl of substrate solution (0.42 mM of TMB (3,3',5,5' tetramethyl benzidine) and 3.2 mM of H_2O_2 in 50 mM sodium acetate solution) was added to each well followed by gentle shaking for 0.2 hour. Fifty µl of stopping solution (0.5 M sulfuric acid) was then added to stop the enzymatic reaction. After gently shaking for 0.1 hour, plates were read by an ELISA reader at dual wavelength of 450 and 600 nm. Two-fold dilution of positive control serum from 1: 100 to 1: 3200 with 1% BSA was applied to each plate. Relative negative controls were also included within each plate which included normal serum control (replaces serum sample with serum from non-autoimmune mice), detection antibody control (no sample antibody added), plate control (no DNA coating and sample antibody) and substrate control (no DNA coating, sample antibody and detection antibody). Diluted serum samples were compared with in-plate positive control antibody to determine positive anti-DNA antibody ratio. In each ELISA plate, a serial dilution of positive control serum was applied and the reading of half the 1600 x dilution was arbitrarily chosen as the cut off point to determine positive anti-DNA antibody.

Statistical Analysis. Body weight, feed intake, feed efficiency and survival days were compared by Student's t-test to determine the treatment effect. Positive proteinuria ratio and positive anti-DNA IgG antibody ratio were analyzed by Fisher's exact test using a SAS computer program version 5.

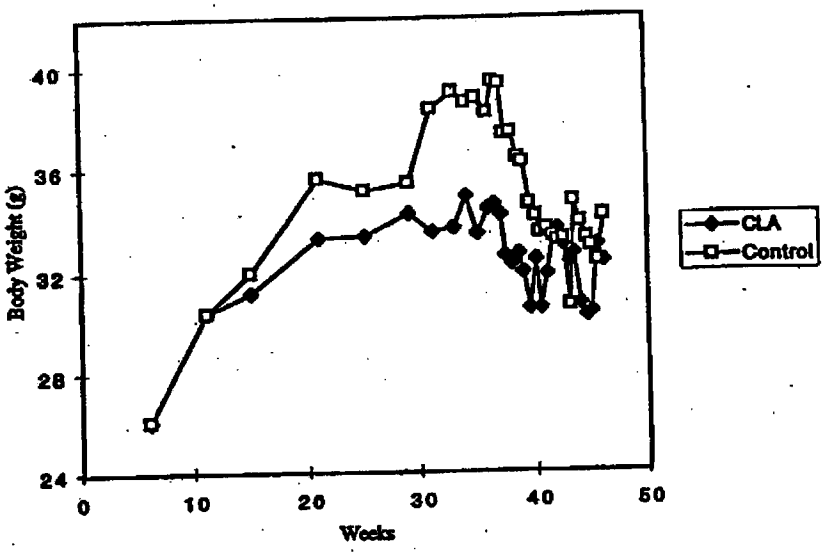


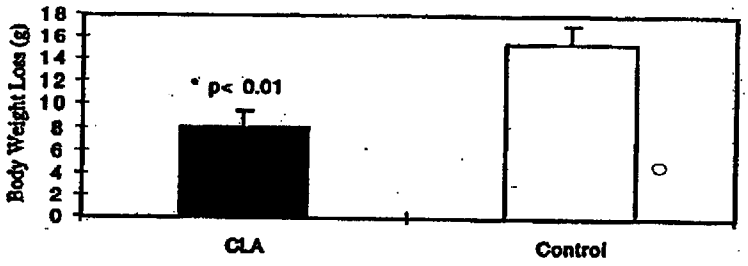
Fig. 1. Effect of dietary CLA on body weight. Body weight was not significantly different between two dietary treatments until 31 week old. Significant differences ($p < 0.05$) between CLA and control groups observed between week 31 to 39. After 39 weeks, no differences were observed. CLA = mice fed 0.5% CLA supplemented diet. Control = mice fed 0.5% corn oil supplement diet.

RESULTS

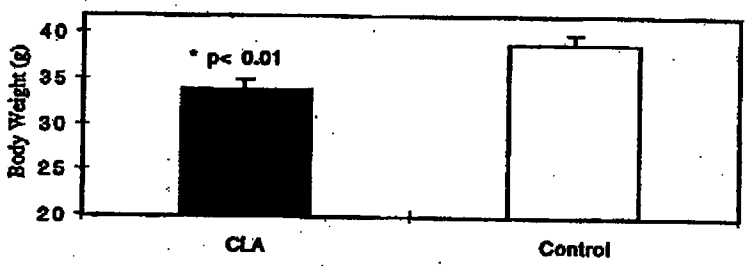
Body Weight and Food Intake. Dietary treatments had no influence on food intake for the first three weeks (CLA 161.4 g, control 166.2 g). Body weight was not affected by CLA feeding until 31 weeks of age, when the control group was found to be significantly heavier than the CLA group (Fig. 1). These differences continued until mice were 39 weeks of age, but not thereafter due to weight loss in control fed mice.

Even though control fed mice were heavier than CLA fed mice during weeks 31 to 39 (Fig. 1), dietary CLA reduced weight loss after the onset of proteinuria (Fig. 2a). The control group was heavier in body weight than the CLA group at

a. Mean body weight loss between proteinuria onset and death



b. Mean body weight at proteinuria onset



c. Mean body weight at last measure before death

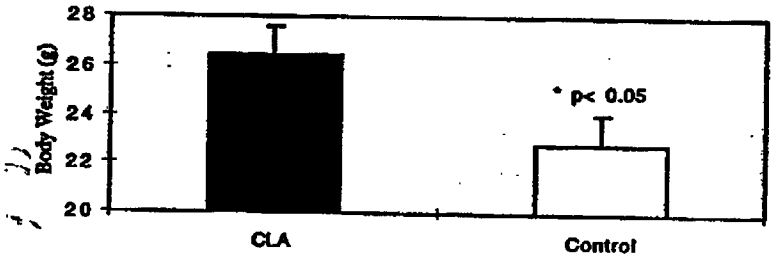


Fig.2. Effect of dietary CLA on body weight, body weight changes between proteinuria onset and death. Data are presented as Mean + SEM. SEM: Standard error of each of above means; standard error based on pooled estimate of variance from ANOVA. In (a) and (c), sample size for CLA and control groups are 7 and 6 respectively. The treatment effect is significant at $p < 0.01$ for (a) and $p < 0.05$ for (c). In (b), sample size for CLA and control groups are 10 and 8 respectively. The treatment effect is significant at $p < 0.01$. CLA = mice fed 0.5% CLA supplemented basal diet. Control = mice fed 0.5% corn oil supplement diet.

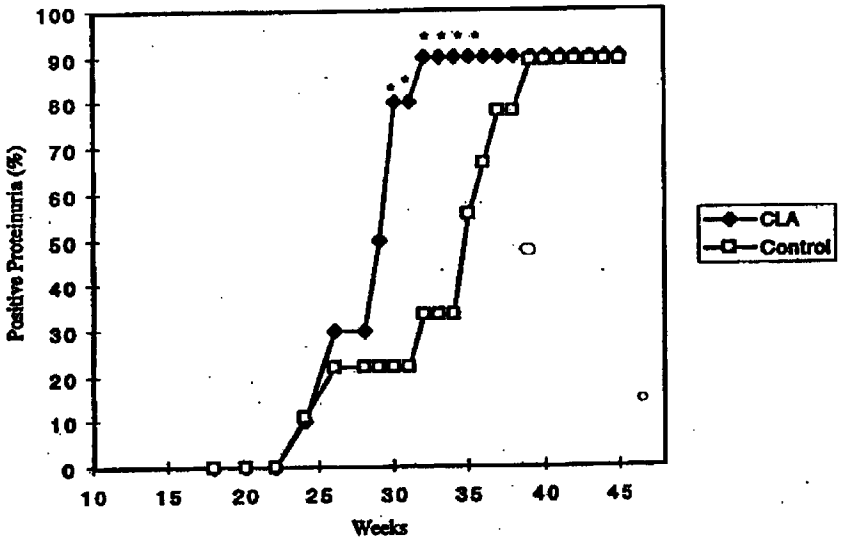


Fig 3. Effect of dietary CLA on the incidence of positive proteinuria in NZB/W F1 mice. Positive proteinuria was determined as more than 1000 μg/ml of protein in urine as described in Research Design and Methods. CLA = mice fed 0.5% CLA supplemented diet. Control = mice fed 0.5% corn oil supplement diet. * $p < 0.05$ when compared between CLA and control groups at a specific time.

the onset of proteinuria (Fig. 2b). However, by the time the disease had progressed to death, the CLA group was significantly heavier than the control group (Fig. 2c). Body weight loss after proteinuria onset until death in the control group was twice that of the CLA group (Fig. 2a). Body weight loss post proteinuria onset was not a consequence of shorter duration for CLA group. Actually, survival days after proteinuria onset was 49% longer for the CLA (88 days) than the control group (59 days) ($p < 0.05$).

Proteinuria. A minimum concentration designation of 1,000 μg/ml of urine protein was chosen to be the criteria of positive proteinuria (11, 14). The basal level of urine protein concentration was about 350 μg/ml in the mice prior to the

presence of anti-DNA antibodies. CLA fed mice had a significantly greater incidence of proteinuria than control fed mice between weeks 27 to 35, but not thereafter ($p < 0.05$) (Fig. 3).

Anti-DNA Antibodies. The percentage of mice with positive anti-ds DNA IgG antibodies was not significantly different between the groups except at week 14 when the CLA group had a higher percentage of positive serum anti-dsDNA Ab (Fig. 4a). Positive serum for anti-single stranded DNA IgG antibodies (anti-ssDNA Ab) appeared earlier than dsDNA Ab but was not influenced by dietary treatment (Fig. 4b).

Survival Rate and Day. The average life spans of CLA (296 days) and control (302 days) groups were not significantly different. However, percent survival was lower in the CLA group at some points between day 260 to day 320 (Fig. 5).

Summary of the Effect of CLA on NZB/W F1 Mice in the Development of Autoimmunity. The time line in Figure 6 shows that the CLA group tended to develop anti-ss DNA and anti-ds DNA antibodies before the control fed mice. Proteinuria onset occurred significantly earlier in the CLA fed group than the control group. While no difference was observed in the average days of survival (CLA: 296 days, control: 302 days), days of survival post onset of proteinuria was significantly (49 %) longer in the CLA fed mice when compared to the control fed mice (Fig. 6).

DISCUSSION

Conjugated linoleic acid has been shown to enhance lymphocyte blastogenesis (6, 7), and enhance select immunoglobulin production (15). Immune modulating

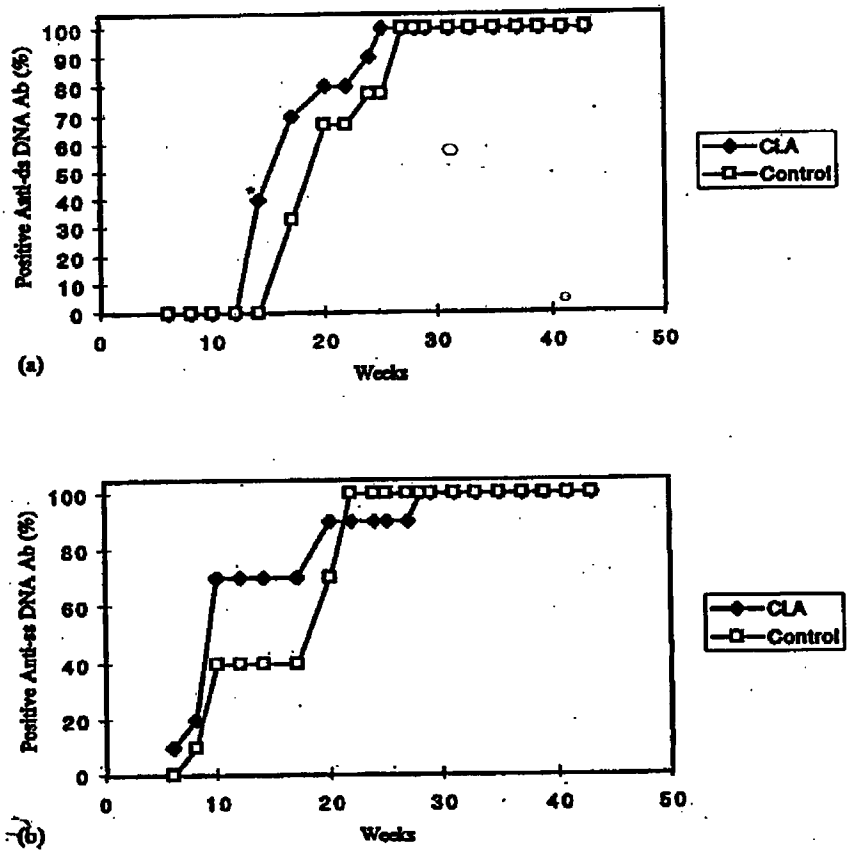


Fig. 4. Effect of dietary CLA on the percentage of positive anti-DNA antibody. Positive antibody titer was determined by comparing each sample with in-plate positive control antibody. In each ELISA plate, a serial dilution of positive control serum was applied and the reading of half the 1600 x dilution was arbitrarily chosen as the cut off point to determine positive anti-DNA antibody. Serum samples were diluted 80 x, and all duplicate samples from the same time point were run in the same plate (29, 30). (a) Positive anti-ds DNA antibody percentage (b) Positive anti-ss DNA antibody percentage. CLA = mice fed 0.5% CLA supplemented diet. Control = mice fed 0.5% corn oil supplement diet. * $p = 0.054$

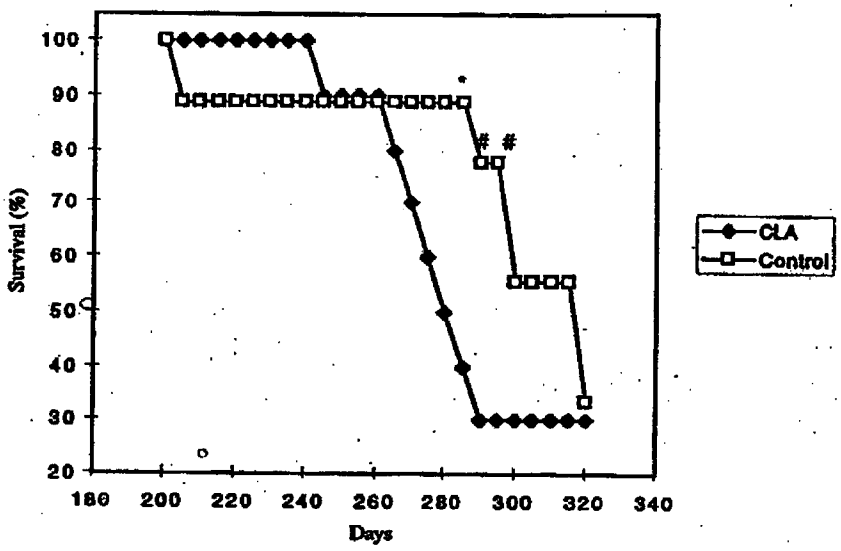


Fig. 5. Effect of CLA on survival rate in NZB/W F1 mice. CLA = mice fed 0.5% CLA supplement diet. Control = mice fed 0.5% corn oil supplement diet. * $p = 0.04$, # $p = 0.051$.

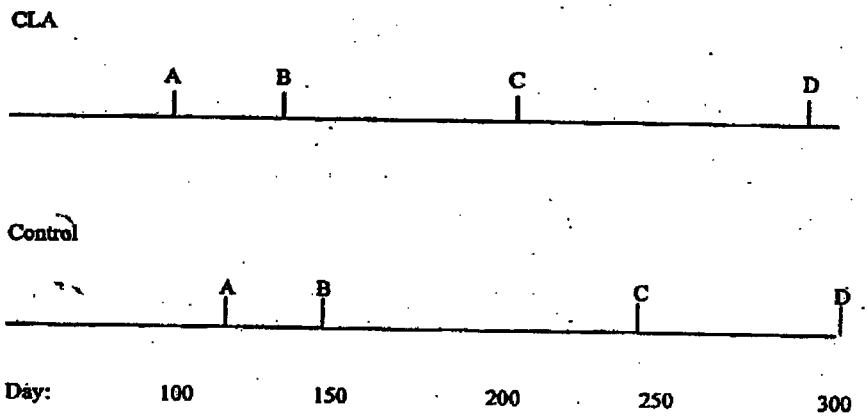


Fig. 6. Summary of the effect of CLA on NZB/W F1 mice in the development of autoimmunity. A: the average day of positive anti-ss DNA Ab (CLA: 99, control: 115). B: the average day of positive anti-ds DNA Ab (CLA: 130, control: 149). C: the average day of positive proteinuria (CLA: 208, control: 242). D: the average day of survival (CLA: 296, control: 302). Proteinuria showed up faster in CLA group than in control group, however, CLA group had a longer survival after proteinuria onset ($p < 0.05$).

activity of CLA would suggest that it would alter the events of autoimmune disease. However, in this study, NZB/W F1 mice, prone to develop anti-nuclear antibodies and succumb to renal failure, presumably to immune complexes, were not more susceptible when fed CLA than control fed mice. Another immune enhancing nutrient, α-tocopherol, showed that immune modulating abilities of a nutrient do not necessarily predict the course or nature of autoimmune disease (16). Vitamin E fed in excess of the level to prevent deficiency has been shown to enhance both humoral and cell-mediated immunities (17). When supplemented to lupus patients, vitamin E did not aggravate the autoimmune symptom (18). On the other hand, omega-3 fatty acids attenuated or delayed the onset of autoimmune disease in NZB/W F1 mice (10). These examples, and the one provided in this study, suggest that the immune modulating activity of a compound does not necessarily predict the outcome of an autoimmune disorder.

Conjugated linoleic acid did result in the production of anti-nuclear antibodies and proteinuria at an early period of life. However, the appearance of renal failure (hence death) was not accelerated in time. Actually, the time between the development of proteinuria and death was longer in the CLA fed mice than the control fed mice. These results suggest that CLA may actually protect the autoimmune mice from renal failure associated with immune complex disorders.

The reduced body weight in the CLA group relative to the control group was consistent with the data of others (19). However, CLA-fed mice lost less weight between the onset of proteinuria and death than control-fed mice. Body weight at the time of death was therefore greater in CLA-fed mice than control-fed mice. These results are not explained by the length of time between proteinuria and death, since CLA-fed mice lived an average of 22 days longer post proteinuria onset than control fed mice. The extended length of life after proteinuria in CLA-fed mice may be related to CLA's protection against end-stage autoimmune-related emaciation. CLA has been shown to protect against immune induced

cachexia (5, 7, 20). Mice, chicks, and rats lost significantly less weight post endotoxin injection when fed CLA as compared to those receiving supplemental linoleic acid. A possible mechanism by which CLA prevents immune related cachexia is through altered lipoxygenase and cyclooxygenase products (5). Decreased prostaglandins and leukotrienes in animal tissues from CLA fed animals has been demonstrated (21, 22).

CLA protection against weight loss may be the result of the reduction in macrophage cytokine production associated with dietary CLA (23). Tumor necrosis factor (TNF)-α, a potent macrophage cytokine, plays a major role in causing extensive body weight loss in chronic inflammation (24). Lower production of TNF-α has been shown in NZB/W F1 mice. Regular injections of TNF-α in NZB/W F1 retarded the onset and reduced the severity of glomerulonephritis (25). While a decreased TNF-α production or a decreased response to TNF-α (26) caused by dietary CLA provides a reasonable explanation for protection against body weight loss, despite exacerbation of autoimmune disease, it fails to explain prolonged survival post proteinuria onset.

In SLE patients, Th1/Th2 cytokine imbalance has been reported with abnormally high Th2 cytokines and low Th1 cytokine activity (27). In animal models, more complete studies have been done to show the relationship between SLE disease progression and cytokine profile change. The development of experimentally inducible SLE in mice seems to involve two stages: increased Th1 cytokines followed by increased Th2 cytokines. The increased Th1 cytokines might be important to disease induction while the increased Th2 cytokines production correlates well with disease progression to the end stage of the disease (28). Sugano et al. (22) showed dietary CLA increased IgA, IgG and IgM in both mouse serum and cultured lymph node cells, while IgE was reduced. An immunoglobulin class switch from IgM to IgG1 then to IgE requires IL-4, a potent Th2 cytokine. Their work suggested CLA enhanced Th1 cytokine production and

caused the Th2 cytokines to be inhibited. CLA's potential effects on cytokine profile may have played a role in extending days of survival post proteinuria onset. Study of such a hypothesis appears warranted.

ACKNOWLEDGEMENTS

This study was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

Corresponding author's address: Department of Animal Sciences, University of Wisconsin-Madison, 260 Animal Sciences, 1675 Observatory Drive, Madison, WI 53706-1284. Phone: (608) 262-7747. Fax: (608) 262-5157. Email: cook@facstaff.wisc.edu.

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Immunoglobulin and Cytokine Production from Spleen Lymphocytes Is Modulated in C57BL/6J Mice by Dietary *Cis*-9, *Trans*-11 and *Trans*-10, *Cis*-12 Conjugated Linoleic Acid

Masao Yamasaki,[1] Hitomi Chujo, Akira Hirao, Nami Koyanagi, Takeaki Okamoto, Naomi Tojo, Ayana Oishi, Toshio Iwata,* Yoshie Yamauchi-Sato,* Takaya Yamamoto,* Kentaro Tsutsumi,* Hirofumi Tachibana and Koji Yamada

*Laboratory of Food Chemistry, Division of Applied Biological Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Higashi-ku, Fukuoka 812-8581, Japan and *Rinoru Oil Mills, Chuo-ku, Tokyo, 103-0027, Japan*

ABSTRACT We evaluated the effect of *cis*-9, *trans*-11 (9*c*,11*t*) and *trans*-10, *cis*-12 (10*t*,12*c*) conjugated linoleic acid (CLA) on the immune system in C57BL/6J mice. Mice were fed experimental diets containing 0% CLA (controls), 1% 9*c*,11*t*-CLA, 1% 10*t*,12*c*-CLA or a 1:1 mixture (0.5% + 0.5%) of these two CLA isomers for 3 wk. Relative spleen weights of all CLA fed mice were greater than the controls. Spleen lymphocytes isolated from the mice fed 10*t*,12*c*-CLA produced more immunoglobulin (Ig)A and IgM but not IgG when stimulated with concanavalin A (ConA) compared with controls. IgA production from unstimulated spleen lymphocytes was greater in the 10*t*,12*c*-CLA group than in controls. Conversely, 9*c*,11*t*-CLA did not affect the production of any of the Ig subclasses. Lymphocytes isolated from 9*c*,11*t*-CLA fed mice produced more tumor necrosis factor-α than the control group. The proportion of B cells in the spleen lymphocyte population was significantly lower in the 9*c*,11*t*-CLA group, and higher in the 10*t*,12*c*-CLA group than in the controls. Compared with the control group, the percentage of $CD4^+$ T cells was lower in the 10*t*,12*c*-CLA group, and the percentage of $CD8^+$ T cells was higher in the 9*c*,11*t*-CLA group. Furthermore, the percentage of $CD8^+$ T cells was higher in the 1:1 mixture group than in controls. The $CD4^+/CD8^+$ ratio was lower in the 1:1 mixture group than in controls. These results suggest that 9*c*,11*t* and 10*t*,12*c*-CLA can stimulate different immunological effects and that the simultaneous intake of the two isomers can change the T cell population. J. Nutr. 133: 784–788, 2003.

KEY WORDS: • *conjugated linoleic acid* • *immunoglobulin* • *mice* • *cytokine*

Conjugated linoleic acid (CLA)[2] is a generic term for the positional and structural isomers of octadecadienoic acid. CLA has been reported to exert various beneficial physiologic effects. In previous reports, the effects of CLA were often evaluated using a mixture of CLA isomers, which contained mainly the *cis*-9, *trans*-11 (9*c*,11*t*) and the *trans*-10, *cis*-12 (10*t*,12*c*)-CLA isomers. Recently, the role of each of these CLA isomers has been studied separately. In fact, some differences between the two isomers have been reported, especially the antiobesity effect (1–4). We reported previously that dietary CLA enhanced immunoglobulin (Ig) production in rat spleen and mesenteric lymph node lymphocytes (5,6). Stimulation of Ig production by dietary CLA was detected at an extremely low dietary level (0.5 g/kg diet) (7). However, determining which of the CLA isomers stimulates Ig production and the mechanism of that stimulation remain to be more clearly elucidated. Thus, the objective of this study was to evaluate the individual and simultaneous effect of 9*c*,11*t* and 10*t*,12*c*-CLA on the production of Ig and cytokines and on the population of B and T cells in spleen lymphocytes from C57BL/6J mice.

MATERIALS AND METHODS

Experimental animals and diet. Semipurified 9*c*,11*t* and 10*t*,12*c*-CLA and safflower oil were purchased from Rinoru Oil Mills (Nagoya, Japan). Male 4-wk-old C57BL/6J mice (n = 40) (Japan CLEA, Tokyo, Japan) consumed a nonpurified commercial diet and water ad libitum for 2 wk after their arrival. After acclimation, the mice were divided into 4 groups of 10 mice each. They were kept in the Biotron Institute of Kyushu University with a 12-h light:dark cycle (light, 0800–2000 h) in an air conditioned room (20°C and 60% humidity under specific pathogen-free conditions). This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government. The experimental diets were manufactured according to the AIN-93G standard (8) and contained 0 g/kg CLA (control), 5 g/kg 9*c*,11*t* and 5 g/kg 10*t*,12*c*-CLA (1:1 mixture), 10 g/kg 9*c*,11*t* (9*c*,11*t*) or 10*t*,12*c*-CLA (10*t*,12*c*) (**Table 1**). For the basic dietary fat source, we used high linoleic acid safflower oil as in

[1] To whom correspondence should be addressed.
E-mail: masawo@agr. kyushu-u.ac.jp.

[2] Abbreviations used: ConA, concanavalin A; CLA, conjugated linoleic acid; Ig, immunoglobulin; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; PE, phycoerythrin; POD, peroxidase; TNF-α, tumor necrosis factor-α.

Manuscript received 24 September 2002. Initial review completed 9 October 2002. Revision accepted 25 November 2002.

TABLE 1

Composition of the experimental diet[1]

Ingredient	g/kg diet
Corn Starch	397.5
Casein	200.0
Dextrinized corn starch	132.0
Sucrose	100.0
Fat[2]	70.0
Cellulose	50.0
AIN-93G mineral mix	35.0
AIN-93G vitamin mix	10.0
L-Cystine	3.0
Choline bitartrate	2.5
Tert-buthylhydroquinone	0.014

1 Experimental diet was prepared according to the AIN-93G guidelines (8).
2 Detailed fatty acid composition in each dietary group is shown in TABLE 2.

our previous report (7). The fatty acid composition of these diets is shown in **Table 2**. At the end of the feeding period, mice were killed by drawing blood from the abdominal aorta under light anesthesia with diethylether. Immediately after excision, each tissue was weighed and the lymphocytes were isolated from the spleen.

Preparation and cultivation of spleen lymphocytes. Preparation of spleen lymphocytes was performed according to the method described previously (5) without removing adhesive cells such as macrophages and mononuclear cells. First, a cell suspension prepared from the spleen was rinsed with RPMI 1640 medium three times (Nissui, Tokyo, Japan). Then, 5 mL of the cell suspension was added to Lympholyte-mouse (Cedarlane, Hornby, Canada) to isolate the lymphocytes and the suspension was again washed three times with RPMI 1640 medium. The lymphocytes, 2.5×10^9 cells/L, were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Intergen, Purchase, NY) with or without 5 mg/L concanavalin A (ConA) (Sigma, St. Louis, MO) and incubated at 37°C for 24 h.

Measurement of immunoglobulin levels. Measurement of Ig concentration in the cultured medium was performed by a sandwich ELISA. Rabbit anti-mouse IgA (Zymed, San Francisco, CA), goat anti-mouse IgG (H+L) (Zymed), rabbit anti-mouse IgM (μ-chain specific) (Zymed), and anti-mouse IgE from clone LO-ME-3 (heavy-chain specific) (Technopharm Biotechnology, Paris, France) were used to fix each Ig. These antibodies were diluted using 10% Block Ace (Dainihon Pharmaceutical, Osaka, Japan), added to a 96-well plate and incubated for 1 h at 37°C. Then, 300 μL of 10% (25% in IgE) Block Ace was added and kept at 4°C overnight; samples (50 μL) were added to each well for 1 h at 37°C. Each well was treated with a solution of either peroxidase (POD)-conjugated goat anti-mouse IgA (Zymed), POD-conjugated goat anti-mouse IgG (H+L) (Zymed), POD-conjugated rabbit anti-mouse IgM (Zymed), or POD-conjugated goat anti-mouse IgE [GAM/IgE (Fc) PO, Nordic Immunological Laboratory, Tilburg, Netherlands] to detect the respective Ig and incubated for 1 h at 37°C (20 min, 4°C for IgE). The plates were rinsed with PBS containing 0.5 g/L polyethylene sorbitan monolaurate (Nacalai Tesque, Kyoto, Japan) between each step. Then, a 10:9:1 mixture of 1.8 mmol/L H_2O_2 in 0.2 mol/L citrate buffer (pH 4.0), H_2O, and 11.7 mmol/L of 2,2′-azinobis (3-ethylbenzothiazoline sulfonic acid) was added. Finally, absorbance at 415 nm was measured after the addition of 160 mmol/L oxalic acid to stop the coloring reaction.

Measurement of cytokine levels. We measured the level of interleukin (IL)-2, 4, 5, tumor necrosis factor (TNF)-α and interferon (IFN)-γ in the supernatants of spleen lymphocytes cultured with ConA. IL-2, 4 and 5 were measured using commercial ELISA kits following the manufacturers' experimental protocols (Mouse IL-2, 4 and 5 ELISA kit, BioSource International, Camarillo, CA). TNF-α and IFN-γ levels were measured by sandwich ELISA as previously reported (9). Briefly, rabbit anti-mouse/rat IFN-γ (BioSource) and anti-mouse TNF-α (Endogen, Woburn, MA) (500 times dilution) were used to fix IFN-γ and TNF-α for 1 h at 37°C. Then, blocking was performed using 25% Block Ace at 37°C for 1 h. In the following step, 50 μL of appropriate cultured supernatant was added to each well and incubated for 2 h at 37°C; the plate was then treated with either a diluted solution of biotinylated anti-mouse IFN-γ (Genzyme, Cambridge, MA) (500 times dilution) or biotinylated anti-mouse/rat TNF-α (Genzyme) (250 times dilution) for 1 h at 37°C. After that, streptavidin POD-conjugated (Zymed) diluted by 10% Block Ace was added to each well. Plate washing between each step and the coloring reaction were performed as in the Ig measurement protocol.

B and T cell population analysis. The cell surface expression of CD45R (as a B cell marker) and CD4 and CD8 (as T cell subpopulation markers) was analyzed by flow cytometry. After the isolation of lymphocytes from the spleen, cells were washed with RPMI-1640 medium three times and treated with PBS containing 3% bovine serum albumin for 1 h at 37°C. Cells were divided into two groups (1.0×10^6 cells each); one was exposed to rat phycoerythrin (PE)-conjugated monoclonal anti-mouse CD45R (clone RA3–6B2, Caltag Laboratories, Burlingame, CA) and the other was double stained with rat fluorescein isothiocyanate–conjugated monoclonal anti-mouse CD4 (clone CT-CD4, Caltag Laboratories) and rat PE-conjugated monoclonal anti-mouse CD8b (clone CT-CD8b, Caltag Laboratories). All of the antibody reactions were performed on ice for 1 h, and cells were washed three times with PBS after the antibody treatment. Samples were subjected to flow cytometry (FACS Calibur, Becton Dickinson, Sunnyvale, CA) and a total of 10^4 cells were analyzed to determine the percentage of CD45R-, CD4- and CD8-positive lymphocytes.

Statistical Analysis. At first, data were analyzed using one-way (Tables 3, 5 and 6) or two-way (Table 4) ANOVA. The latter was used to identify differences due to diet or lymphocyte stimulation status. Fisher's Protected Least Significant Difference test was used to determine which means differed ($P < 0.05$). All data are presented as means ± SEM.

RESULTS

Food intakes and body and organ weights. Food intake did not differ among the dietary groups (**Table 3**). Final body weight was higher in the 9*c*,11*t* group than in the 1:1 mixture or 10*t*, 12*c* groups, but it did not differ significantly from the controls. Relative liver weight was greater in the 1:1 mixture and 10*t*,12*c* groups than in the control and 9*c*,11*t* groups. Relative spleen weight was significantly higher in all CLA

TABLE 2

Fatty acid composition of the diets[1]

Groups	Control	1:1 MIX	9c, 11*t*	10*t*, 12*c*
	g/100 g total fatty acids			
Fatty acids				
16:0	6.4	5.5	5.4	5.5
18:0	2.5	2.1	2.1	2.1
18:1	15.9	14.6	15.2	13.9
18:2 (n-6)	72.8	62.5	61.6	62.3
CLA				
9c, 11*t*	ND	6.2	12.5	0.5
10*t*, 12*c*	ND	6.2	0.8	12.5
9c, 11*c*	ND	0.1	ND	0.2
10*c*, 12*c*	ND	0.2	ND	0.3
All *trans*	ND	0.3	0.1	0.5
Others	2.4	2.1	2.1	2.1

1 High linoleic acid safflower oil was the basic dietary fat source. Experimental diets containing 0 g/kg CLA (control), 5 g/kg 9c, 11*t* and 5 g/kg 10*t*, 12*c*-CLA (1:1 MIX), 10 g/kg 9c, 11*t* (9c, 11*t*) or 10*t*, 12*c*-CLA (10*t*, 12*c*). ND, not detected.

TABLE 3

Food intake, body weight and relative organ weights in C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c	ANOVA
			g/day		
Food intake	3.0 ± 0.0	2.9 ± 0.2	2.9 ± 0.1	2.9 ± 0.1	NS
			g		
Body weight					
Initial	22.7 ± 0.5	22.7 ± 0.4	22.9 ± 0.2	22.6 ± 0.3	NS
Final	26.5 ± 0.5^{ab}	26.0 ± 0.5^{b}	27.7 ± 0.3^{a}	25.6 ± 0.6^{b}	$P < 0.05$
			g/100 g body		
Organ weights					
Liver	42.4 ± 1.0^{b}	51.9 ± 1.0^{a}	44.2 ± 1.1^{b}	56.9 ± 3.2^{a}	$P < 0.001$
Spleen	2.9 ± 0.2^{b}	3.4 ± 0.3^{a}	3.2 ± 0.2^{a}	3.5 ± 0.5^{a}	$P < 0.01$
Lung	6.0 ± 0.1	6.3 ± 0.1	5.7 ± 0.2	6.4 ± 0.2	NS
Heart	5.3 ± 0.1	5.5 ± 0.1	5.6 ± 0.2	5.6 ± 0.2	NS
Kidney	13.3 ± 0.2	13.7 ± 0.1	13.5 ± 0.4	13.2 ± 0.4	NS
PWAT	4.1 ± 0.4^{a}	1.4 ± 0.2^{b}	4.2 ± 0.4^{a}	1.1 ± 0.1^{b}	$P < 0.001$
EWAT	16.1 ± 1.2^{a}	3.5 ± 0.1^{b}	17.1 ± 1.1^{a}	1.8 ± 0.2^{b}	$P < 0.001$

[1] Values are mean ± SEM, $n = 10$. Means in a row not sharing a superscript letter differ, $P < 0.05$. PWAT, perirenal white adipose tissue; EWAT, epididymal white adipose tissue; CLA, conjugated linoleic acid.
[2] The "1:1 MIX" denotes a 1:1 mixture of 9c, 11t and 10t, 12c-CLA.

groups than in the control group. Perirenal and epididymal white adipose tissue weights were significantly lower in the 1:1 mixture and 10t,12c compared with the control and 9c,11t groups. Relative lung, heart and kidney weights did not differ among the dietary groups.

Immunoglobulin production from the spleen lymphocytes. ConA did not affect IgA productivity in spleen lymphocytes (**Table 4**). IgA productivity of 10t,12c-fed mice was approximately twice that ($P < 0.05$) of the control group in the presence of ConA. IgA productivity also differed between the control and 10t,12c groups without ConA stimulation. IgA production from the lymphocytes in the 9c,11t group was lower than that of the 10t,12c group, irrespective of ConA stimulation. ConA stimulation significantly elevated IgG productivity in spleen lymphocytes. IgM production in spleen lymphocytes was significantly modulated by the type of dietary fat but not by ConA stimulation. IgM production from the lymphocytes in the 10t,12c group was significantly higher than that of the control group, irrespective of ConA stimulation. The 9c,11t and control groups did not differ significantly in IgM productivity. IgM productivity for the 1:1 mixture group was intermediate between the 9c,11t and 10t,12c groups. IgE production by lymphocytes was not affected by diet or ConA stimulation.

Cytokine productivity. Cytokines were not detected in any of the cultured supernatants from lymphocytes that were not stimulated with ConA. No significant differences were found in IL-2, 4, 5 and IFN-γ production among any of the dietary groups (data not shown). TNF-α production of spleen lymphocytes from mice in the 9c,11t group was significantly higher than that of the 10t,12c and control groups (**Table 5**).

B- and T-cell population. The percentage of B cells in the 9c,11t group was significantly lower than in the control group, whereas the 10t,12c group was higher (**Table 6**). The value for the 1:1 mixture group was intermediate between the 9c,11t and 10t,12c groups. The percentage of $CD4^+$ T cells was significantly lower in the 1:1 mixture and 10t,12c groups than in the control group. The percentage of $CD8^+$ T cells was the highest for the 1:1 mixture group followed by the 9c,11t group. The $CD4^+/CD8^+$ ratios in the 9c,11t and 10t,12c groups were

TABLE 4

Immunoglobulin A, G, M and E production of spleen lymphocytes isolated from C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c
		ConA (+),[3] μg/L		
IgA	3.0 ± 0.4^{a}	3.4 ± 0.5^{ab}	2.8 ± 1.6^{a}	6.2 ± 1.7^{b}
IgG	35.0 ± 13.5	31.1 ± 4.0	31.1 ± 6.1	32.9 ± 7.6
IgM	10.7 ± 2.6^{a}	13.4 ± 1.4^{ab}	10.9 ± 2.5^{a}	19.0 ± 3.1^{b}
IgE	18.4 ± 0.7	17.0 ± 1.2	19.4 ± 0.4	18.3 ± 0.7
		ConA (−), μg/L		
IgA	2.1 ± 0.4^{ab}	2.0 ± 0.2^{ab}	1.3 ± 0.8^{a}	4.6 ± 1.5^{b}
IgG	19.2 ± 5.2*	15.7 ± 4.6*	20.2 ± 5.3	31.2 ± 8.5
IgM	12.1 ± 2.8^{a}	15.3 ± 2.4^{ab}	13.7 ± 3.9^{ab}	22.7 ± 4.2^{b}
IgE	17.4 ± 1.0	18.3 ± 1.3	18.4 ± 0.5	18.9 ± 0.5

	Two-way ANOVA			
	IgA	IgG	IgM	IgE
ConA	NS	$P < 0.05$	NS	NS
Diet	$P < 0.05$	NS	$P < 0.05$	NS
Interaction	NS	NS	NS	NS

[1] Values are mean ± SEM, $n = 5$. Means in a row not sharing superscript letter differ, $P < 0.05$. * Different from Con A-stimulated, $P < 0.05$.
[2] The "1:1 MIX" means a 1:1 mixture of 9c, 11t and 10t, 12c-conjugated linoleic acid.

TABLE 5

Tumor necrosis factor-α production of spleen lymphocytes isolated from C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c	ANOVA
			ng/L		
TNF-α	411 ± 68[a]	474 ± 32[ab]	574 ± 76[b]	388 ± 21[a]	$P < 0.05$

[1] Values are mean ± SEM, $n = 5$. Means in a row not sharing a superscript letter differ, $P < 0.05$.
[2] The "1:1 MIX" denotes a 1:1 mixture of 9c, 11t and 10t, 12c-conjugated linoleic acid.

lower than in the control group, and the ratio in the 1:1 mixture group was lower than any of the other dietary groups. The $CD4^+/CD8^+$ ratios in the 9c,11t and 10t,12c groups were also significantly lower than the control group.

DISCUSSION

The aim of this study was to evaluate the isomer-specific effect of CLA on the function of spleen lymphocytes in C57BL/6J mice. At present, 9c,11t and 10t,12c CLA are recognized for various beneficial physiologic functions, with each CLA isomer having both individual and synergistic roles in carrying out those functions such as a body fat–reducing effect or a growth-promoting effect. For example, 10t,12c-CLA definitely plays a leading part in reducing body fat (1,10), and we confirmed that this isomer does indeed have a specific body fat–reducing effect in mice (Table 3). On the other hand, 9c,11t-CLA has been reported to promote mouse growth (10), which our present data also support because we found that the final body weight in the 9c,11t group was significantly higher than that in the control group (Table 3). In addition, dietary CLA significantly increased the spleen weight compared with the control group; however, there was no significant difference among the CLA-fed mice. These data suggest that 9c,11t and 10t,12c-CLA have almost the same ability to increase the spleen mass and that no synergistic effect exists between these isomers. In previous reports, CLA feeding did not increase spleen weight in Sprague-Dawley rats (5,7). Thus, we speculate that species specificity exists between rats and mice spleens in terms of sensitivity to dietary CLA.

We reported previously that dietary CLA could enhance IgA, IgG and IgM production from the rat mesenteric lymph node and spleen lymphocytes (5,6). In rat spleen lymphocytes, only a diet containing 0.5 g/kg CLA dramatically promoted IgA, IgG and IgM production (7). However, as far as we know, no report has clearly shown which isomer(s) promote Ig production. In the present study, significant enhancement of IgA and IgM production was detected in the 10t,12c group but not in the 1:1 mixture and 9c,11t groups. This result indicates that 10t,12c-CLA plays a leading part in promoting Ig production. To examine the effect of CLA on the B cell ratio in spleen lymphocytes, we measured the amount of B lymphocyte–specific surface marker CD45R present to determine the positive cell population percentage. As a result, the B-cell percentage in the 10t,12c group was significantly higher than the control level. On the other hand, a decrease in the B-cell percentage was observed in the 9c,11t group, and the level in the 1:1 mixture group was intermediate to the 9c,11t and 10t,12c groups. Judging from these results, the elevation of the B-cell percentage in spleen lymphocytes by 10t,12c-CLA might be counteracted by 9c,11t-CLA. Taken together, it is likely that elevation of the B-cell ratio by 10t,12c-CLA contributes in part to the promotion of Ig production in spleen lymphocytes. However, we must consider that this augmentation of Ig production (~×2) may be due in part to increased production per B cell because the increased number of $CD45R^+$ cells was not doubled.

Significant enhancement of IgA and IgM production was also detected in the 10t,12c group with or without ConA, which is a T lymphocyte–specific mitogen (Table 4). However, IgA and IgM production by spleen lymphocytes did not increase with ConA stimulation. We reported previously that dietary CLA enhanced Ig production in rat spleen and mesenteric lymph node lymphocytes with LPS stimulation which is a B lymphocyte–specific mitogen (6). In a previous report, dietary CLA stimulated IL-2 productivity in mouse spleen lymphocytes or splenocytes (11,12). In this study, neither dietary 9c,11t nor 10t,12c-CLA could inhibit IL-2 production from spleen lymphocytes. In addition, dietary 9c,11t and 10t,12c-CLA did not affect IL-4 and 5 production from spleen lymphocytes stimulated with ConA, and none of these cytokines were detected without ConA. These data indicate that the enhancement of Ig production in spleen lymphocytes by 10t,12c-CLA was not modulated by the stimulation of T lymphocytes during a 24-h incubation period.

Conversely, 9c,11t-CLA significantly stimulated TNF-α production, and this result is consistent with our previous data (9). Turek et al. (13) reported that dietary CLA reduced TNF-α and IL-6 production in rat macrophages, and CLA has been reported to suppress TNF-α related cachexia (14). Conversely, recent reports showed that CLA did not affect TNF-α production in splenocytes isolated from tumor-bearing rats stimulated with *Escherechia coli* endotoxin (15). Unfortu-

TABLE 6

B and T lymphocytes population of spleen lymphocytes isolated from C57BL/6J mice fed 0 g/kg CLA, 5 g/kg 9c, 11t and 5 g/kg 10t, 12c-CLA, 10 g/kg 9c, 11t, or 10t, 12c-CLA diet for 3 wk[1]

	Control	1:1 MIX[2]	9c, 11t	10t, 12c	ANOVA
$CD45R^+$, %	52.6 ± 0.4[c]	55.6 ± 0.5[b]	50.1 ± 0.5[d]	60.2 ± 0.1[a]	$P<0.001$
$CD4^+$, %	21.3 ± 0.4[a]	19.1 ± 0.1[b]	21.8 ± 0.2[a]	18.3 ± 0.2[c]	$P<0.001$
$CD8^+$, %	10.7 ± 0.1[c]	12.7 ± 0.4[a]	11.8 ± 0.2[b]	11.1 ± 0.1[c]	$P<0.01$
$CD4^+/CD8^+$	2.0 ± 0.0[a]	1.5 ± 0.1[d]	1.9 ± 0.0[b]	1.7 ± 0.0[c]	$P<0.001$

[1] Values are mean ± SEM, $n = 5$. Means in a row not sharing superscript letter differ, $P < 0.05$.
[2] The "1:1 MIX" denotes a 1:1 mixture of 9c, 11t and 10t, 12c-conjugated linoleic acid.

nately, there is little information concerning isomer specificity in the regulation of TNF-α production. Our present data indicate that 9*c*,11*t* and 10*t*,12*c*-CLA have quite different effects on the production of TNF-α in spleen lymphocytes, but further studies are warranted to elucidate the target immune cells of each CLA isomer.

Most $CD8^+$ T cells are major histocompatibility complex class I restricted killer T cells and exert cytotoxic activity when they are activated. CLA has been reported to elevate the $CD8^+$ T cell population of porcine peripheral mononuclear cells (16,17). Conversely, CLA elevated $CD4^+$ T cell population in mice and chicks (14,18), which resulted in an elevation of the $CD4^+/CD8^+$ ratio. In this report, only 10*t*,12*c*-CLA decreased the $CD4^+$ T cells ratio and only the 9*c*,11*t*-CLA elevated the $CD8^+$ T cells ratio. Interestingly, the $CD4^+/CD8^+$ ratio was the lowest in the 1:1 mixture group. These results suggest that both CLA isomers cooperatively modulated the T cell subpopulation and also acted individually.

In summary, 10*t*,12*c*-CLA increases IgA and IgM production and 9*c*,11*t*-CLA increases TNF-α production. Moreover, these CLA isomers synergistically reduce the $CD4^+/CD8^+$ T cell population ratio.

ACKNOWLEDGMENT

We thank Perry Seto for proofreading the manuscript.

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Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

Michihiro Sugano[a,b,*], Akira Tsujita[a], Masao Yamasaki[a], Miwa Noguchi[a], and Koji Yamada[a]

[a]Laboratory of Food Science, Kyushu University School of Agriculture, Fukuoka 812-8581, and [b]Faculty of Human Life Sciences, Prefectural University of Kumamoto, Kumamoto 862-8502, Japan

ABSTRACT: The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB_4, lung LTC_4 and serum prostaglandin E_2 levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.
Lipids 33, 521–527 (1998).

Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1–4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E_2 (PGE_2), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE_2 level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al.* (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

MATERIALS AND METHODS

Preparation of CLA. CLA was prepared according to the method described by Ip *et al.* (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

*To whom correspondence should be addressed at Faculty of Human Life Sciences, Prefectural University of Kumamoto, Tsukide 3-1-100, Kumamoto 862-8502, Japan. E-mail: suganomi@pu-kumamoto.ac.jp.

Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas–liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm × 60 m, film thickness, 0.25 μm; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography–mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9*c*,11*t*/9*t*,11*c*, 29.8; 10*t*,12*c*, 29.6; 9*c*,11*c*, 1.3; 10*c*,12*c*, 1.4; 9*t*,11*t*/10*t*,12*t*, 18.6; linoleic acid, 5.6; and others, 13.7.

Animals and diets. The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

Preparation of peritoneal exudate cells (PEC). The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5.6 mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at 200 × *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

TABLE 1
Fatty Acid Composition of Dietary Fat[a]

Fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

[a]Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).

Measurement of leukotriene B_4 (LTB_4) and histamine. LTB_4 was measured as described elsewhere (20–22). PEC (2×10^6 cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of PGB_2 (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at −20°C for 30 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant was filtered through a 4-GV 0.22 μm filter (Millipore Corp., Tokyo, Japan). LTB_4 was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150 × 6.0 mm, 5 μL particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM CH_3COONH_4 and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of LTB_4 was achieved by comparing the peak area of LTB_4 with that of PGB_2. Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

Preparation of spleen and mesenteric lymph node (MLN) lymphocytes. Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of 2.5×10^6 cells/mL with or without 2.5 μg/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations of IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

T-cell population analysis. Spleen and MLN lymphocytes were analyzed as $CD4^+$- and $CD8^+$-cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luto, United Kingdom).

Measurement of serum and culture supernatant Ig by ELISA. Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')2, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 μL of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300 μL of the blocking solution overnight at 4°C, each well was treated with 100 μL of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100 μL of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100 μL of POD-conjugated rabbit anti-rat IgG (Fab')2 (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100 μL of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween 20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100 μL of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Betyl, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means ± SE.

RESULTS

Growth performance and tissue weight. As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

Release of chemical mediators from PEC. PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB_4 were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

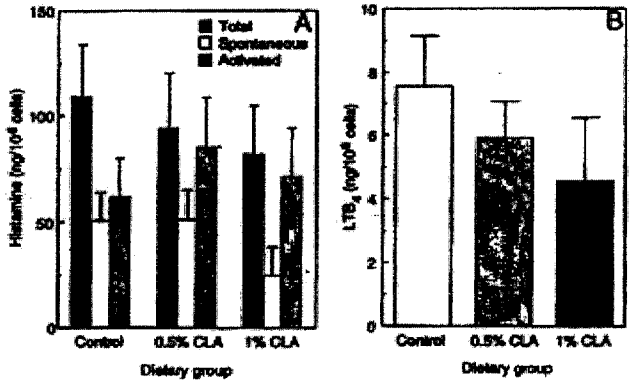

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B_4 (LTB_4) release (B) in rat peritoneal exudate cells. Means ± SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB_4 release in response to the dietary level of CLA, but the difference was not significant.

Tissue eicosanoid levels. The effect of CLA on LTB_4 and LTC_4 levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB_4, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC_4 level was observed. However, the concentration of LTC_4 in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB_4 also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE_2 are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE_2, while there was no effect of CLA on the splenic level of PGE_2.

Fatty acid compositions of PEC and splenic lymphocyte lipids. The PUFA composition of PEC and spleen lympho-

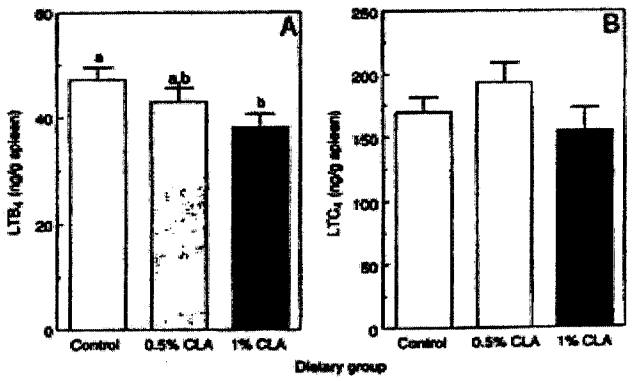

FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB_4 and (B) leukotriene C_4 (LTC_4). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

TABLE 2
Effects of CLA on Growth and Tissue Weights of Rats[a]

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09[a]	4.11 ± 0.09[a,b]	4.54 ± 0.07[b]
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07[a]	1.09 ± 0.09[a,b]	0.97 ± 0.14[b]
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

[a]Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PEC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

Serum thiobarbituric acid value. The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

Serum Ig levels. As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

Ig levels in spleen and MLN lymphocytes. Table 4 shows the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

Subsets of MLN lymphocytes. The proportion of T-lymphocyte populations of MLN was analyzed as $CD4^+$ and $CD8^+$ subsets. There were no effects of CLA on their relative proportions ($CD4^+/CD8^+$ ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

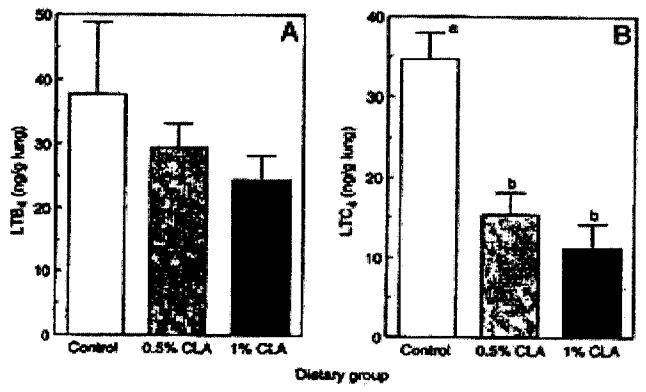

FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB_4 and (B) LTC_4. Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figures 1 and 2.

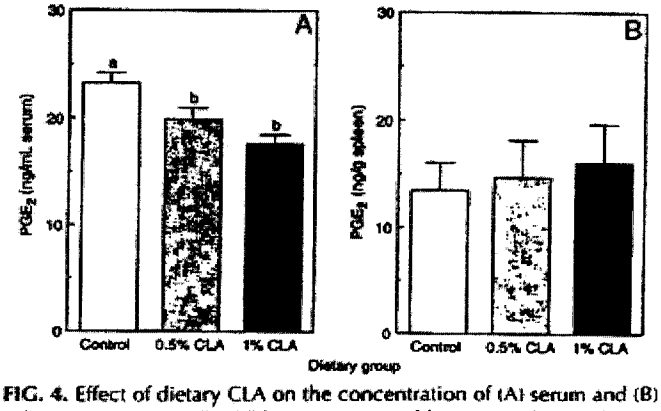

FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E_2 (PGE_2). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For other abbreviation see Figure 1.

TABLE 3
Effects of CLA on Polyunsaturated Fatty Acid Compositions of Peritoneal Exudate Cells and Spleen Lymphocyte Total Lipids of Rats[a]

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
	(wt%)		
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9*t*,11*c*/9*c*,11*t*	n.d.	0.1	0.2
10*t*,12*c*	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8[a]	15.4 ± 1.3[a,b]	14.7 ± 1.7[b]
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9*t*,11*c*/9*c*,11*t*	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10*t*,12*c*	n.d.	0.2 ± 0.0	0.2 ± 0.0

[a]Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$; n.d., not detected. For other abbreviation see Table 1.

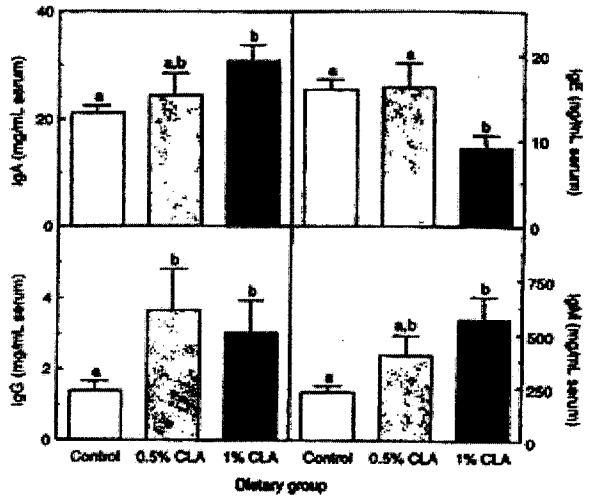

FIG. 5. Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common lettter (a,b) are significantly different at $P < 0.05$. For abbreviation see Figure 1.

DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production *in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB_4, LTC_4, and PGE_2: CLA significantly reduced LTC_4 production in the lung but not in the spleen. A similar tissue-specific reduction of LTC_4 was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB_4 but not LTC_4 was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2–4). Eicosanoid production is known to be dependent on

TABLE 4
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats[a]

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.8 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9[A]	348 ± 8[B]	394 ± 6[C]
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13[a]	4.78 ± 1.77[b]	5.05 ± 0.10[b]	2.91 ± 0.23[A]	8.72 ± 0.90[B]	22.3 ± 0.7[C]
IgG	n.d.	3.08 ± 0.69[a]	28.1 ± 4.38[b]	n.d.	4.64 ± 0.11[A]	31.9 ± 4.1[B]
IgM	1.86 ± 0.34[a]	4.74 ± 0.50[a]	96.6 ± 13.4[b]	2.85 ± 0.44[A]	6.36 ± 0.48[B]	122 ± 9[C]
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17[A]	4.52 ± 0.29[A]	3.74 ± 0.21[B]

[a]Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at $P < 0.05$. The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al.* (37) observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig levels preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

ACKNOWLEDGMENTS

The authors thank Dr. S. Samman of the Department of Human Nutrition Unit, The University of Sydney, Australia for his valuable criticism during the preparation of this manuscript. This study was supported by a Grant-in-Aid for Scientific Research B from the Ministry of Education, Culture and Science of Japan.

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[Received November 24, 1997, and in final revised form and accepted April 9, 1998]